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1 **The biosynthetic pathway of ubiquinone contributes to pathogenicity of *Francisella***

2
3 Running title: *Ubiquinone and Francisella pathogenicity*

4 Katayoun Kazemzadeh¹, Mahmoud Hajj Chehade¹, Gautier Hourdoir¹, Camille Dorothée
5 Brunet¹, Yvan Caspar^{2,3}, Laurent Loiseau⁴, Frederic Barras^{5,6}, Fabien Pierrel¹ and Ludovic
6 Pelosi^{1*}

7
8 From ¹CNRS, CHU Grenoble Alpes, Grenoble INP, TIMC, Université Grenoble Alpes,
9 Grenoble, France; ²Laboratoire de Bactériologie-Hygiène Hospitalière, Centre National de
10 Référence des *Francisella*, Centre Hospitalier Universitaire Grenoble Alpes, 38000, Grenoble,
11 France; ³Univ. Grenoble Alpes, CHU Grenoble Alpes, CEA, CNRS, IBS, F-38000 Grenoble,
12 France; ⁴Aix Marseille Université, CNRS, Laboratoire Chimie Bactérienne, Institut
13 Microbiologie de la Méditerranée, 31 Chemin Joseph Aiguier, Marseille 13009, France;
14 ⁵SAMe Unit, Department of Microbiology, Institut Pasteur, Paris, France and ⁶IMM-UMR
15 2001 CNRS-Institut Pasteur, Paris, France.

16
17 *To whom correspondence should be addressed to ludovic.pelosi@univ-grenoble-alpes.fr

18
19 **Keywords:** ubiquinone biosynthesis, coenzyme Q, quinone, aerobic respiration, *Francisella*
20 *tularensis*, *Francisella novicida*.

21
22 **Abstract**

23 *Francisella tularensis* is the causative agent of tularemia. Because of its extreme
24 infectivity and high mortality rate, this pathogen was classified as a biothreat agent.
25 *Francisella* spp are strict aerobe and ubiquinone (UQ) has been previously identified in these
26 bacteria. While the UQ biosynthetic pathways were extensively studied in *Escherichia coli*
27 allowing the identification of fifteen Ubi-proteins to date, little is known about *Francisella*
28 spp. In this study, and using *Francisella novicida* as a surrogate organism, we first identified
29 UQ₈ as the major quinone found in the membranes of this bacterium. Then, we characterized
30 the UQ biosynthetic pathway in *F. novicida* using a combination of bioinformatics, genetics
31 and biochemical approaches. Our analysis disclosed the presence in *Francisella* of ten
32 putative Ubi-proteins and we confirmed eight of them by heterologous complementation in *E.*
33 *coli*. The UQ biosynthetic pathways from *F. novicida* and *E. coli* share a similar pattern.

34 However, differences were highlighted: the decarboxylase remains unidentified in *Francisella*
35 spp and homologs of the Ubi-proteins involved in the O₂-independent UQ pathway are not
36 present. This is in agreement with the strictly aerobic niche of this bacterium. Then, *via* two
37 approaches, i.e. the use of an inhibitor (3-amino-4-hydroxybenzoic acid) and a transposon
38 mutant, which both strongly impair the synthesis of UQ, we demonstrated that UQ is essential
39 for the growth of *F. novicida* in a respiratory medium and contributes to its pathogenicity in
40 *Galleria mellonella* used as an alternative animal model.

41

42 **Importance**

43 *Francisella tularensis* is the causative bacterium of tularemia and is classified as a
44 biothreat agent. Using multidisciplinary approaches, we investigated the ubiquinone (UQ)
45 biosynthetic pathway that operates in *F. novicida* used as a surrogate. We showed that UQ₈ is
46 the major quinone identified in the membranes of *Francisella novicida*. We identified a new
47 competitive inhibitor, which strongly decreased the biosynthesis of UQ. Our demonstration of
48 the crucial role of UQ for the respiratory metabolism of *F. novicida* and for the involving in
49 its pathogenicity in the *Galleria mellonella* model should stimulate the search for selective
50 inhibitors of bacterial UQ biosynthesis.

51

52 **Keywords**

53 *Francisella novicida*, *Francisella tularensis*, ubiquinone biosynthesis, respiratory chain,
54 metabolism, pathogenicity.

55

56 **Introduction**

57 *Francisella tularensis* is a Gram-negative, strictly aerobic, facultative intracellular
58 pathogen responsible for tularemia. Infection can occur by inhalation, ingestion, transmission
59 from arthropod vectors or exposure to infected animals (1). After its entry into macrophages,
60 the bacteria are sequestered into phagosomes and prevent further endosomal maturation.
61 *Francisella* cells then disrupt the phagosome and are released into the cytosol in which they
62 rapidly proliferate (2). Eventually, the infected cells undergo apoptosis or pyroptosis, and the
63 progeny bacteria are released to initiate new rounds of infection (2). Currently, there is no
64 suitable vaccine against tularemia and due to its extreme infectivity and high virulence, *F.*
65 *tularensis* species have been classified as a biothreat agent (3). The genus *Francisella*
66 includes the three species: *F. tularensis*, *F. novicida* and *F. philomiragia* (4). Moreover, *F.*

67 *tularensis* is further divided into the subspecies *tularensis* (Type A strains) and *holarctica*
68 (Type B strains), which are the most virulent strains responsible for human disease, whereas
69 *F. philomiragia* and *F. novicida* are avirulent in healthy humans (4). A *F. novicida* type strain
70 U112 is commonly used as a surrogate for *Francisella tularensis* in virulence studies using
71 animal models (5).

72 The development of genome-scale genetic methods allowed the identification of
73 hundreds of genes participating to variable extents to *Francisella* virulence (6). However, the
74 specific contribution of only a limited number of these genes was demonstrated at the
75 molecular level. Although an important proportion of the identified genes are related to
76 metabolic functions, the relationship between metabolism and the life cycle of *Francisella* is
77 still poorly understood. However, global analysis of genes essential for the growth in culture
78 of *F. novicida* U112 (7) and more recently of that of *F. tularensis* ssp *tularensis* Schu S4 (8)
79 highlighted the involvement of several ubiquitous pathways found in proteobacteria. Among
80 the most significant are the folate pathway, the heme synthesis pathway, the methylerythritol
81 phosphate pathway involved in isoprenoid synthesis, the chorismate pathway and the
82 ubiquinone (UQ) synthesis pathway, on which this work is focused.

83 Isoprenoid quinones are conserved in most respiratory and photosynthetic organisms
84 and function primarily as electron and proton carriers in the electron-transfer chains.
85 Quinones are composed of a polar redox-active head group linked to a lipid side chain, which
86 varies in both length and degree of saturation (9). Proteobacteria contain two main types of
87 quinone, i.e. benzoquinones and naphthoquinones, represented by UQ (or coenzyme Q) and
88 menaquinone (MK)/ demethylmenaquinone (DMK), respectively (9). UQ is the major
89 electron carrier used for the reduction of dioxygen by various cytochrome oxidases, whereas
90 MK and DMK function predominantly in anaerobic respiratory chains (9). However, as
91 demonstrated recently in *Pseudomonas aeruginosa*, UQ can also be produced and used as a
92 main respiratory quinone under anaerobic conditions (10). Besides its role in bioenergetics,
93 UQ was also reported to be involved in gene regulation, oxidative stress, virulence and
94 resistance to antibiotics (11, 12). More recently, new functions for UQ in bacteria were
95 discovered such as its requirement for *Escherichia coli* to grow on medium containing long-
96 chain fatty acids as a carbon source (13). UQ biosynthesis in aerobic conditions has been
97 widely studied in *E. coli* (14). The classical UQ biosynthetic pathway requires twelve proteins
98 (UbiA to UbiK and UbiX). UbiC catalyzes the first committed step in the biosynthesis of UQ,
99 the conversion of chorismate to the 4-hydroxybenzoate (4HB) precursor. Then, UbiA, UbiD

100 to UbiI and UbiX catalyze the prenylation, decarboxylation, hydroxylations and methylations
101 of the phenyl ring of the 4HB to synthesize UQ. In addition, UbiB and UbiK are accessory
102 proteins while UbiJ is involved in the assembly and/or the stability of the aerobic Ubi-
103 complex, which was recently characterized in *E. coli* (15). This latter is able to synthesize UQ
104 also under anoxic conditions and we identified three proteins, UbiU, UbiV and UbiT, which
105 are required for UQ biosynthesis only under anoxic conditions (16).

106 Here, we show that UQ₈ is the major quinone of *F. novicida* U112. We identified
107 candidate Ubi-proteins in *F. novicida* U112 and validated their functions by heterologous
108 complementation in *E. coli* mutant strains. Our results show that UQ biosynthesis in
109 *Francisella* spp is mostly similar to that of *E. coli*, with the notable absence of UbiX and
110 UbiD for the decarboxylation step. Genetic and chemical inactivation of UQ biosynthesis
111 thanks to a transposon mutant and to a new inhibitor (3-amino-4-hydroxybenzoic acid),
112 respectively, demonstrated that UQ₈ is crucial for the growth of *F. novicida* in respiratory
113 media and that UQ deficiency impairs the pathogenicity of *F. novicida* against *Galleria*
114 *mellonella*. Altogether, our results shed light on the role of UQ in the life cycle of *Francisella*
115 and show that UQ contributes to its pathogenicity.

116

117 **Results**

118 **UQ₈ is the major quinone of *F. novicida*.**

119 The quinone content of *F. novicida* grown under ambient air at 37°C in Chamberlain
120 media supplemented with either glucose (fermentative medium) or succinate (respiratory
121 medium) as the only carbon source was determined and compared with that of *E. coli*
122 MG1655 grown in the same fermentative medium. In the electrochromatograms of lipid
123 extracts from *F. novicida*, a single peak was observed around 8.3 min, the same retention time
124 as UQ₈ in *E. coli* extracts (Fig. 1A). Note that in these analyses, UQ₁₀ was used as an internal
125 standard, which was added to the samples. MS analysis of the major peak in *F. novicida*
126 extracts showed a predominant ammonium adduct ($M^+ NH_4^+$) with an m/z ratio of 744.5,
127 together with minor adducts, such as Na⁺ (749.7) and H⁺ (727.8) (Fig. 1B). These masses
128 identify UQ₈ (monoisotopic mass, 726.5) as the major quinone produced by *F. novicida*.
129 Interestingly, the carbon source in the culture media did not greatly affect the UQ₈ content
130 (Fig. 1A). The *F. novicida* extracts did not contain any naphthoquinones, unlike *E. coli*, which
131 showed predominantly demethylmenaquinone (DMK₈) eluting around 12 min. The absence of
132 detectable levels of naphthoquinones in *F. novicida* lipid extracts (Fig. 1A) is in agreement

133 with the absence of menaquinone biosynthesis (Men or futasoline) encoding genes in its
134 genome. Together, our results establish that *E. coli* and *F. novicida* share UQ₈ as a main
135 quinone in aerobic conditions.

136

137 **Identification of Ubi-proteins in the *Francisella* spp.**

138 To identify candidate Ubi-proteins in *F. novicida*, UbiX, and UbiA to UbiK from *E.*
139 *coli* MG1655 were screened for homologs in the protein sequence data set, available at
140 MicroScope (www.genoscope.cns.fr/agc/microscope), using the BLASTP software. As listed
141 in Table S1, this analysis identified eight homologous proteins in *F. novicida*, i.e. UbiA to
142 UbiC, UbiE, UbiG to UbiI and UbiK, called hereafter UbiA_{*Fn*} to UbiC_{*Fn*}, UbiE_{*Fn*}, UbiG_{*Fn*} to
143 UbiI_{*Fn*} and UbiK_{*Fn*}, respectively. Genes *ubiA_{Fn}* and *ubiC_{Fn}* in one hand and genes *ubiI_{Fn}* and
144 *ubiH_{Fn}* in another hand present an organization similar to the *ubiC-ubiA* and the *ubiH-ubiI*
145 operons from *E. coli*, respectively (12). As reported previously for *Pseudomonas aeruginosa*
146 (17) and *Xanthomonas campestris* (18), *F. novicida* possesses a Coq7 hydroxylase, which is a
147 functional homolog of the UbiF protein found in *E. coli* and other species (19). The detection
148 of an homolog for *E. coli* UbiJ required less restrictive Blast parameters. We noticed that the
149 gene coding for the putative UbiJ candidate *FTN_0460*, hereafter called *ubiJ_{Fn}*, lies between
150 *ubiE_{Fn}* and *ubiB_{Fn}*, an organization similar to the *ubiE-ubiJ-ubiB* operon from *E. coli* (20).
151 UbiJ_{*Fn*} has 21% amino-acid identity with UbiJ from *E. coli* and both proteins contain a sterol
152 carrier protein 2 domain in their N-terminal regions (<http://pfam.xfam.org/>) (21). The same
153 Ubi-proteins were identified in the highly virulent strain *F. tularensis* ssp *tularensis* Schu S4
154 (Table S1).

155 Homologs of UbiD and UbiX were not yet identified and the counterparts of these two
156 proteins in *Francisella* spp remain to be determined. The work is in progress in our
157 laboratory. Under anaerobic conditions, *E. coli* still synthesizes UQ, and we recently
158 identified three genes, which we called *ubiT*, *ubiU*, and *ubiV*, as essential for this process
159 (16). Homologs of *ubiT*, *ubiU*, and *ubiV*, which participate to the O₂-independent UQ
160 biosynthetic pathway, were not identified in the screened *Francisella* genomes (Table S1), in
161 agreement with the strictly aerobic metabolism of *Francisella* spp. In overall, these data show
162 that the O₂-dependent UQ biosynthetic pathways in *F. novicida*, in *F. tularensis* and in *E. coli*
163 are related, the major difference being the absence of UbiX-UbiD for the decarboxylation step
164 (Fig. 2).

165

166 **Functional characterization of *Ubi_{Fn}*-proteins in *E. coli*.**

167 To test whether the candidate Ubi-proteins identified in *F. novicida* were indeed
 168 involved in UQ biosynthesis, we assessed their capacity to functionally complement *E. coli*
 169 strains in which the UQ protein-encoding genes were inactivated ($\Delta ubiAc$, $\Delta ubiBc$, $\Delta ubiCc$,
 170 $\Delta ubiEc$, $\Delta ubiFc$, $\Delta ubiGc$, $\Delta ubiHc$, $\Delta ubiIc$, $\Delta ubiJ$ and $\Delta ubiKc$, see Table S2). We assessed the
 171 quinone content and the capacity to grow on solid minimal medium containing fermentable
 172 (glucose) or respiratory (succinate) carbon sources. *E. coli* $\Delta ubiAc$, $\Delta ubiBc$, $\Delta ubiGc$, $\Delta ubiHc$
 173 and $\Delta ubiJ$ transformed with empty vector are unable to synthesize UQ₈ (Fig. 3A) and are thus
 174 unable to grow on a respiratory medium (Fig. 3C). In contrast, their growth on a fermentative
 175 medium is not affected (Fig. 3C). Except for the $\Delta ubiAc$ mutant strain, in which the
 176 prenylation reaction of the 4HB is impaired, most mutants accumulate an early intermediate
 177 corresponding to octaprenylphenol (OPP) (Fig. 2 and 3A). *E. coli* $\Delta ubiEc$ and $\Delta ubiFc$ cells
 178 accumulate C2-demethyl-C6-demethoxy-UQ₈ (DDMQ₈) and C6-demethoxy-UQ₈ (DMQ₈),
 179 which are the substrates of UbiE and UbiF, respectively (Fig. 2 and 3A). We found that
 180 $UbiA_{Fn}$, $UbiB_{Fn}$, $UbiE_{Fn}$, $Coq7_{Fn}$ and $UbiG_{Fn}$ restored growth of *E. coli* $\Delta ubiAc$, $\Delta ubiBc$,
 181 $\Delta ubiEc$, $\Delta ubiFc$ and $\Delta ubiGc$ cells on respiratory medium (Fig. 3C) and allowed for UQ₈
 182 biosynthesis in LB medium to 96, 26, 7, 49 and 38% of the level of UQ₈ present in the WT
 183 cells, respectively (Fig. 3A and 4A). Concomitantly, OPP content decreased and $Coq7_{Fn}$
 184 abolished the accumulation of DMQ₈ in $\Delta ubiFc$ cells (Fig. 3A). As we previously reported, *E.*
 185 *coli* $\Delta ubiIc$ and $\Delta ubiKc$ cells displayed a strong decrease in UQ₈ (22, 23), but the residual
 186 UQ₈ content was sufficient to support growth on succinate (Fig. 3A and 3C). Similar results
 187 were obtained with $\Delta ubiCc$ cells grown in minimal M9 medium (Fig. 3B and 3C), which had
 188 to be used instead of LB since the later contains 4HB that restores normal UQ₈ content in
 189 $\Delta ubiCc$ (data not shown). In all three strains, the expression of the corresponding Ubi-
 190 proteins, $UbiC_{Fn}$, $UbiI_{Fn}$ and $UbiK_{Fn}$, increased significantly the UQ₈ content (Fig. 4A and
 191 4B). Since the increase obtained in $\Delta ubiIc$ cells was moderate (from 25 to 40%), we further
 192 confirmed the ability of $UbiI_{Fn}$ to catalyze *C5-hydroxylation* by using an *E. coli* $\Delta ubiIc\Delta ubiF$
 193 strain. This deletion mutant lacks C5- and C6-hydroxylation activities and consequently
 194 accumulates 3-octaprenyl-4-hydroxyphenol (4HP₈) (22). We found that $UbiI_{Fn}$ was able to
 195 restore DMQ₈ biosynthesis in *E. coli* $\Delta ubiIc\Delta ubiF$ cells (Fig. S1), i.e. to catalyze C5-
 196 hydroxylation, concomitantly to a strong decrease of 4HP₈. Taken together, all these results
 197 confirm unambiguously that $UbiA_{Fn}$, $UbiB_{Fn}$, $UbiC_{Fn}$, $Coq7_{Fn}$, $UbiE_{Fn}$, $UbiG_{Fn}$, $UbiI_{Fn}$ and

198 UbiK_{F_n} are the functional counterpart of the *E. coli* Ubi-proteins and we propose that they
199 compose the biosynthetic pathway of UQ₈ in *F. novicida*. Only two proteins, UbiJ_{F_n} and
200 UbiH_{F_n} did not complement the *E. coli* Δ ubiJ and Δ ubiHc (Fig. 3A, 3C and 4A). The low
201 percentage of identity between UbiJ and UbiH from *E. coli* and their homologs in *F. novicida*
202 (21 and 27%, respectively) could explain these results (Table S1).

203

204 **UQ₈ biosynthesis is essential for the growth of *F. novicida* in respiratory medium.**

205 To evaluate the physiological importance of UQ for *F. novicida*, we screened *ubi*-
206 genes in the *F. novicida* transposon (Tn) mutant library available at the Manoil Laboratory
207 (7). Only the Tn mutant of *ubiC_{F_n}* (called hereafter Tn-*ubiC_{F_n}*) was available in the library,
208 and we compared this mutant strain to its isogenic control strain U112 (Table S2). Recall that
209 UbiC catalyzes the first committed step in the biosynthesis of UQ, i.e. the conversion of
210 chorismate to 4HB (Fig. 2). First, we showed that the growth of Tn-*ubiC_{F_n}* cells under
211 ambient air in respiratory Chamberlain medium was severely impaired compared to the WT
212 (Fig. 5A). In contrast, the growth of *F. novicida* in fermentative medium was less affected
213 (Fig. 5B). In parallel, UQ₈ content was strongly lowered in Tn-*ubiC_{F_n}* cells from 166 to 7
214 pmol/mg cells in fermentative medium and from 134 to 11 pmol/mg cells in respiratory
215 medium (Fig. 5C). As expected, addition of 4HB to the culture rescued the growth of Tn-
216 *ubiC_{F_n}* in respiratory medium and increased the UQ₈ content to WT levels (Fig. 5A and 5C).
217 Taken together, these results show the overall requirement of UQ₈ for the growth of *F.*
218 *novicida* especially in respiratory medium.

219

220 **3A4HB inhibits UQ₈ biosynthesis and impairs the growth of *F. novicida* in respiratory 221 medium.**

222 Besides genetic inactivation of the UQ pathway, we were interested in the possibility
223 to decrease UQ levels by chemical inhibition. Since we had found UQ to be particularly
224 important for growth of *F. novicida* in respiratory medium (Fig. 5A), we screened for
225 compounds that could inhibit growth in such medium. We tested several compounds: 3-
226 amino-4-hydroxybenzoic acid (3A4HB), 4-amino-benzoic acid (pABA), 4-amino-2-methoxy-
227 benzoic acid (pA2MBA) and 4-amino-3-methoxy-benzoic acid (pA3MBA). All these
228 molecules are analogs of 4HB, the native precursor of UQ (Fig. S2A). We observed that
229 bacterial growth was slightly affected in respiratory medium in presence of pABA and
230 pA2MBA, while pA3MBA inhibited growth both in fermentative and respiratory media (Fig.

231 S2B-C). Interestingly, 3A4HB strongly impaired bacterial growth in respiratory medium,
232 while inhibition was milder in fermentative medium (Fig. S2B-C). Based on these results, we
233 followed up on this compound.

234 We then examined how and to what extent 3A4HB could affect UQ₈ biosynthesis in *F.*
235 *novicida*. Bacteria were cultured under ambient air in fermentative Chamberlain medium
236 supplemented with 3A4HB (from 10 μM to 1 mM, final concentration). Endogenous UQ₈
237 content was measured in the bacterial cells and compared to a control condition in which only
238 DMSO was added. Figure 6A shows that the UQ₈ content decreased with increasing
239 concentrations of 3A4HB in the medium, with 0.5 mM yielding to a ~90% decrease of the
240 UQ₈ content. Concomitantly, we confirmed that growth of *F. novicida* in presence of 1 mM
241 3A4HB was strongly impaired in respiratory medium (Fig. 6B), but less so in a fermentative
242 medium (Fig. 6C). Control experiments showed that addition of 4HB to the growth medium
243 counteracted the negative effect of 3A4HB, both in terms of UQ₈ biosynthesis and bacterial
244 growth (Fig. 6A to 6C).

245 Treatment with 3A4HB caused the accumulation of a redox compound that eluted at
246 6.5 min (compound X on Fig. 6A). MS analysis of this peak showed a predominant proton
247 adduct (M⁺ H⁺) with an m/z ratio of 682.6, together with a minor sodium adduct (M⁺ Na⁺)
248 with an m/z ratio of 704.6 (Fig. 6D). Both species are compatible with a monoisotopic mass
249 of 681.7 g.mol⁻¹, which could correspond to that of 2-octaprenyl-3-methyl-6-amino-1,4-
250 benzoquinone (Fig. 6E). According to the sequence of reactions proposed in Figure 2, the
251 formation of compound X would result from prenylation of 3A4HB, decarboxylation and
252 hydroxylation at C1 and then methylation at C3. Thus, 3-octaprenyl-2-methyl-5-amino-1,4-
253 benzoquinone seems to be the “dead-end” product of the UQ₈ pathway in *F. novicida* cells
254 treated with 3A4HB. Collectively, these results demonstrate unequivocally that 3A4HB acts
255 as a competitive inhibitor of UQ₈ biosynthesis and affects particularly the respiratory
256 metabolism of *F. novicida*.

257

258 **UQ₈ is involved in the pathogenesis of *F. novicida* in the later steps of the infection.**

259 We evaluated the importance of UQ in the pathogenicity of *F. novicida* by studying
260 the Tn-*ubiC_{Fn}* mutant. To assess the overall virulence of the Tn-*ubiC_{Fn}* strain in a whole
261 organism, we used the wax moth (*G. mellonella*) infection model, which was previously used
262 in studies of human pathogenic and closely related opportunistic and non-pathogenic
263 *Francisella* spp, such as *F. novicida* (24-27). We monitored the survival of larvae infected

264 with the Tn-*ubiC_{Fn}* strain or with the isogenic strain U112 as control. When the larvae are
265 turning grey/black and no movement of the larval legs can be observed, they are considered
266 dead (Fig. 7A). The Tn-*ubiC_{Fn}* strain was found to be statistically much less virulent than the
267 wild type, but was nevertheless still capable to kill *Galleria* larvae (Fig. 7B). This result
268 suggests that UQ₈ is involved in the virulence potential of *F. novicida* in *G. mellonella*. To
269 better understand the role of UQ₈ in the different stages of infection in *G. mellonella*, the
270 pathogenicity of the isogenic control strain pre-treated with 1 mM 3A4HB was studied in
271 order to mimic a UQ₈ acute deficiency. Recall that this treatment causes a ~90% decrease of
272 the UQ₈ content (Fig. 6A) but the inhibition should be alleviated over the infection cycle in
273 the larvae where 3A4HB is not present. The pretreatment with 3A4HB has no effect on the
274 capacity of *F. novicida* to kill *Galleria* larvae (Fig. 7C), suggesting that UQ₈ does not
275 contribute to the virulence of *Francisella novicida* in the early steps of the infection but more
276 likely in later ones. This result contrasts with that obtained with the Tn-*ubiC_{Fn}* strain, which
277 represents a chronic deficiency of UQ₈.

278

279 Discussion

280 The chemical analysis performed in this paper established that UQ₈ is the major
281 isoprenoid quinone synthesized by *F. novicida*. In two representative *Francisella* genomes,
282 we identified homologs for nine of the twelve genes, which are currently known to contribute
283 to UQ biosynthesis in *E. coli* under aerobic conditions. We confirmed the function of seven of
284 the nine homologs by heterologous complementation of *E. coli* Δ *ubi* mutants. From these
285 results, we show again that *E. coli* is a good model to study the function of most exogenous
286 *ubi*-genes (19). We could not confirm the function of UbiH_{Fn} and UbiJ_{Fn}, but the fact that
287 *ubiH_{Fn}* and *ubiJ_{Fn}* show the same genetic organization as in *E. coli* (a *ubiI-ubiH* operon and a
288 *ubiE-ubiJ-ubiB* operon) strongly supports the implication of these genes in the UQ
289 biosynthetic pathway. Interestingly, both proteins are part of the Ubi-complex in *E. coli* (15).
290 We hypothesize that the low identity of UbiH_{Fn} and UbiJ_{Fn} with their *E. coli* homologs
291 (~25%) might impair their assembly within the *E. coli* Ubi-complex and thus compromise our
292 *in vivo* complementation assays. Another possibility relates to the proposed implication in UQ
293 biosynthesis of a non-coding RNA partially overlapping the ORF of UbiJ from *E. coli* (28).
294 We note that the expression of UbiJ from *X. campestris* was also unable to complement an *E.*
295 *coli* Δ *ubiJ* strain (18). *Francisella* spp shares with *P. aeruginosa* and *X. campestris* a yeast
296 Coq7 protein homolog, which catalyzes the C6-hydroxylation as UbiF from *E. coli* (17, 18,

297 29). As we demonstrated previously, the Coq7 proteins are found in all three subclasses,
298 alpha-, beta- and gamma-proteobacteria. In contrast, homologs of UbiF proteins are limited to
299 the gamma-proteobacteria (19). Our analysis disclosed also the presence in *Francisella* spp of
300 UbiI and UbiH homologous proteins, which catalyze C5- and C1-hydroxylation in *E. coli*,
301 respectively (22, 30). Consequently, we propose that both *E. coli* and *Francisella* spp share a
302 UQ biosynthetic pathway involving three hydroxylases, i.e. UbiI, UbiH and UbiF in *E. coli* or
303 UbiI, UbiH and Coq7 in *Francisella* spp. Several studies highlighted that the enzymes
304 involved in multiple steps of the UQ biosynthetic pathway vary between bacterial species
305 (14), like for the hydroxylation steps (19), or for the production of 4HB from chorismate by
306 UbiC or XanB2 proteins (31). The decarboxylation step involves UbiD and UbiX in *E. coli*
307 (32), but we could not identify homologs in *Francisella* genomes. A candidate gene *ubiZ* was
308 proposed based on its co-localization with *ubiE* and *ubiB* in the genomes of *Acinetobacter* spp
309 and *Psychrobacter* sp. PRwf-1, which are also devoid of homologs of UbiD and UbiX (33).
310 However, *ubiZ* was not confirmed functionally and this gene is not conserved in *Francisella*
311 genomes. We demonstrated that UbiI proteins from *F. novicida* and from *E. coli* shared the
312 same function, i.e. the catalysis of the first hydroxylation of the OPP, which is the product of
313 the decarboxylation step in *E. coli* (Fig. 2). Consequently, we propose that the
314 decarboxylation step occurring in *F. novicida* also precedes the first hydroxylation of the
315 OPP. Collectively, these data suggest the existence of another decarboxylation system
316 operating in UQ biosynthesis in *Francisella* spp and potentially in other bacteria lacking *ubiX*
317 and *ubiD* (34).

318 To assess the essentiality of the UQ biosynthetic pathway in the respiratory
319 metabolism of *F. novicida*, two different approaches were carried out. First, we showed that a
320 transposon mutation of *ubiC_{Fn}* gene, which decreases 4HB synthesis, impaired the growth of
321 *F. novicida* mainly in respiratory medium. Interestingly, among all the *ubi*-gene identified in
322 *Francisella* genomes, only *ubiC* was mutated in large-scale studies (7, 8). This supports that
323 the other *ubi* genes are essential for the viability of *Francisella* spp and strengthens the idea
324 that UQ is key for the development of these bacteria. We noted that the mutation of the *ubiC*
325 gene affects more severely *F. novicida* than *E. coli* for growth in respiratory media, despite
326 both mutants producing comparable amounts of UQ (~7-8% compared to the WT) (Fig. 4B
327 and 5C). As *E. coli* synthesizes naphthoquinones but *F. novicida* does not, we propose that the
328 milder phenotype of the *E. coli ubiC* mutant results from naphthoquinones participating to
329 aerobic respiration, as previously suggested (35). Second, we tested the effect of structural

330 analogs of 4HB and we showed that 3A4HB impaired the growth of *F. novicida* mainly in
331 respiratory medium in agreement with a strong decrease of UQ₈ biosynthesis. We
332 demonstrated that 3A4HB competes with endogenous 4HB and progresses through several
333 steps of the UQ biosynthetic pathway to form the redox compound X that we propose to be 3-
334 octaprenyl-2-methyl-5-amino-1,4-benzoquinone. As the Tn-*ubiC_{Fn}* strain and the control
335 strain U112 treated with 1 mM 3A4HB yielded both to a ~90% decrease of the UQ₈ content
336 and presented a strong impairment of the growth in a respiratory medium (Fig. 5 and 6), we
337 propose that compound X would not be used as a quinone in the respiratory chain of *F.*
338 *novicida*.

339 We noted that homologs of *ubiT*, *ubiU*, and *ubiV*, which belongs to the O₂-
340 independent UQ biosynthetic pathway characterized in *E. coli* and *P. aeruginosa* (10, 16),
341 were not identified in the screened genomes of *Francisella* spp. This result is in agreement
342 with the strictly aerobic metabolism of these bacteria. Indeed, tricarboxylic acid (TCA) cycle
343 and the UQ-dependent electron-transfer chain, leading to efficient oxidative phosphorylation,
344 take place in *Francisella* spp (36). A possible link between stress defense and the TCA cycle
345 was previously suggested in *Francisella* pathogenesis (37). Unfortunately, the contribution of
346 UQ and the electron-transfer chain to virulence has not been well documented to date in
347 *Francisella* spp. Using *G. mellonella* as infection model at the scale of an entire organism, we
348 demonstrated through the study of the Tn-*ubiC_{Fn}* mutant and the isogenic control strain pre-
349 treated with 3A4HB, that UQ₈ contributes to the virulence of *F. novicida* and more likely in
350 the later steps of the infection, during which the bacteria undergo extensive replication (38).
351 Such a notion support the view that, as other facultative intracellular bacteria, *Francisella* spp
352 are able to use several substrates in order to grow in various environments, such as
353 macrophages. Glycerol via the gluconeogenesis and amino acids were identified as main
354 sources of carbon during intracellular replication of *Francisella* spp in the host cells (36, 39).
355 However, glycerol requires UQ to be efficiently metabolized *via* the ubiquitous enzyme GlpD
356 (40) and amino acids degradation is closely linked to the TCA cycle, which produces reducing
357 equivalents in *Francisella* spp (36). Besides its requirement for bioenergetics, UQ might also
358 contribute to the antioxidant capacity of *Francisella* since it was shown to be a potent lipid
359 soluble antioxidant in *E. coli* (41). During its intracellular life, *Francisella* is exposed to
360 oxidative stress. Indeed, as a defense mechanism for the clearance of phagocytosed
361 microorganisms, both macrophages and neutrophils produce reactive oxygen species, which
362 in turn trigger bacterial killing by causing damage to macromolecules (42, 43). We propose

363 that the reduced content in UQ in the Tn-*ubiC_{Fn}* mutant could therefore affect *F. novicida*'s
364 oxidative defense. This hypothesis is in good agreement with recent data showing that
365 reduced expression of UbiC_{Fn} decreases the resistance of *F. novicida* to oxidative stress (44).
366 In a similar way, we showed previously that UbiE, UbiJ and UbiB proteins were needed in
367 *Salmonella enterica* serovar *Typhimurium* intracellular proliferation in macrophages (21).
368 Collectively, all these data assign a role for Ubi-proteins in bacterial intracellular proliferation
369 and, more generally, highlight the importance of UQ production for bacterial virulence.

370

371 **Materials and methods**

372 **Bacterial strains and growth conditions**

373 All bacterial strains used in this study are listed in Table S2. *F. novicida* U112 was
374 obtained from the Centre National de Référence des *Francisella*, CHU Grenoble-Alpes,
375 France. The transposon mutant Tn-*ubiC_{Fn}* in the *F. novicida* U112 strain was obtained from
376 the Manoil Laboratory, Department of Genome Science, University of Washington (7). Both
377 strains were grown on Polyvitex enriched chocolate agar plates (PVX-CHA, bioMérieux,
378 Marcy L'Etoile, France) incubated at 37°C for 48-72 h. Liquid cultures were carried out at
379 37°C with rotary shaking at 200 rpm in Chamberlain medium (45) supplemented with either
380 glucose or succinate (0.4% (wt/vol) final concentration) as the only carbon source. For growth
381 studies, overnight cultures were used to inoculate a 96-well plate to obtain a starting optical
382 density at 600 nm (OD₆₀₀) of around 0.1 and further incubated under shaking at 37°C.
383 Changes in OD₆₀₀ were monitored every 10 min for 40 h using the Infinite 200 PRO
384 microplate reader (Tecan, Lyon, France). When required, the medium was supplemented with
385 4HB in DMSO at 50-100 µM final concentration, pABA, pA2MBA and pA3MBA in DMSO
386 at 1 mM final concentration or 3A4HB at 10 µM-1 mM, final concentration. For CFU
387 counting, bacteria were suspended in PBS and cell suspensions were serially diluted in PBS.
388 For each sample, 100 µL of at least four different dilutions were plated on PVX-CHA plates
389 and incubated for 72 h at 37°C, and CFU were counted using a Scan 100 Interscience.

390 The *E. coli* Δ *ubiA* and Δ *ubiJ* mutants were constructed as described previously (46).
391 Briefly, the *ubiA::cat* and *ubiJ::cat* mutation was generated in a one-step inactivation of the
392 *ubiA* and *ubiJ* genes. A DNA fragment containing the *cat* gene flanked with the 5' and 3'
393 regions of the *ubiA* and *ubiJ* genes was PCR amplified using pKD3 as a template and
394 oligonucleotides 5'-wannerubiA/3'-wannerubiA and 5'-wannerubiJ/3'-wannerubiJ,
395 respectively (Table S3). The Δ *ubiB* mutant was generated as follows: the *cat* gene was

396 inserted in *ubiB* gene between the two sites NruI at 842 and 1004 pb. Then, *ubiB::cat* was
 397 PCR amplified using oligonucleotides 5'-*xbaIubiB*/3'-*xbaIubiB* (Table S3). Strain BW25113,
 398 carrying the pKD46 plasmid, was transformed by electroporation with the amplified
 399 fragments and *Cat*^r colonies were selected. The replacement of chromosomal *ubi* by the *cat*
 400 gene was verified by PCR amplification in the *Cat*^r clones. *E. coli* K-12 strains JW5713 and
 401 JW2226 from the Keio Collection (47) were used as donors in transduction experiments to
 402 construct the $\Delta ubiC::kan$ and $\Delta ubiG::kan$ mutants of *E. coli* MG1655 strains. The $\Delta ubiA$,
 403 $\Delta ubiB$, $\Delta ubiC$, $\Delta ubiE$, $\Delta ubiG$ and $\Delta ubiK$ strains were cured with pCP20 to yield $\Delta ubiAc$,
 404 $\Delta ubiBc$, $\Delta ubiCc$, $\Delta ubiEc$, $\Delta ubiGc$ and $\Delta ubiKc$ strains, respectively (Table S2). *E. coli* strains
 405 (K12, MG1655 or Top10) were grown on lysogeny broth (LB)-rich medium or in M9
 406 minimal medium (supplemented with glucose or succinate, 0.4% (wt/vol) final concentration)
 407 at 37°C. Ampicillin (100 µg/ml), kanamycin (50 µg/ml), chloramphenicol (35 µg/ml) and
 408 IPTG (100 µM) were added when needed.

409

410 **Cloning, plasmid construction, and complementation assays.**

411 The plasmids and the primers used in this study are listed in Tables S2 and S3
 412 (supplemental material), respectively. All the plasmids produced in this work were verified by
 413 DNA sequencing (GATC Biotech, Konstanz, Germany). The *FTN_0385* (*ubiA_{Fn}*), *FTN_0459*
 414 (*ubiB_{Fn}*), *FTN_0386* (*ubiC_{Fn}*), *FTN_0461* (*ubiE_{Fn}*), *FTN_1146* (*coq7_{Fn}*), *FTN_0321* (*ubiG_{Fn}*),
 415 *FTN_1237* (*ubiH_{Fn}*), *FTN_1236* (*ubiI_{Fn}*), *FTN_0460* (*ubiJ_{Fn}*) and *FTN_1666* (*ubiK_{Fn}*) inserts
 416 were obtained by PCR amplification using the *F. novicida* U112 genome as template and the
 417 oligonucleotides described in Table S3. Inserts were EcoRI-BamHI or EcoRI-HindIII digested
 418 and inserted into EcoRI-BamHI- or EcoRI-HindIII-digested pTrc99a plasmids, respectively,
 419 yielding the *pubiA_{Fn}*, *pubiB_{Fn}*, *pubiC_{Fn}*, *pubiE_{Fn}*, *pcoq7_{Fn}*, *pubiG_{Fn}*, *pubiH_{Fn}*, *pubiI_{Fn}*, *pubiJ_{Fn}*
 420 and *pubiK_{Fn}* plasmids (Table S3). The plasmids were transformed into *E. coli* MG1655 strains
 421 with mutation of the *ubiA*, *ubiB*, *ubiC*, *ubiE*, *ubiF*, *ubiG*, *ubiH*, *ubiI*, *ubiJ* and *ubiK* genes
 422 (single and double mutations, see Table S2), and complementation of the UQ₈ biosynthetic
 423 defect was assessed by both measuring the quinone content and plating serial dilutions onto
 424 solid M9 minimal medium supplemented with glucose or succinate (0.4% (wt/vol) final
 425 concentration) as the only carbon sources and overnight growth at 37°C. Expression of the
 426 Ubi-proteins was induced by addition of IPTG to a final concentration of 100 µM.

427

428 **Lipid extractions and quinone analysis**

429 Cultures (5 ml under ambient air) were cooled down on ice 30 min before
430 centrifugation at 3200 X g at 4 °C for 10 min. Cell pellets were washed in 1 ml ice-cold PBS
431 and transferred to preweighted 1.5 mL Eppendorf tubes. After centrifugation at 12,000 g at 4
432 °C for 1 min, the supernatant was discarded, the cell-wet weight was determined (~5–30 mg)
433 and pellets were stored at -20°C. Quinone extraction from cell pellets was performed as
434 previously described (22). Lipid extracts corresponding to 1 mg of cell-wet weight were
435 analyzed by HPLC electrochemical detection-MS (ECDMS) with a BetaBasic-18 column at a
436 flow rate of 1 mL/min with mobile phases composed of 50% methanol, 40% ethanol, and a
437 mix of 90% isopropanol, 10% ammonium acetate (1 M), and 0.1% TFA). When necessary,
438 MS detection was performed on an MSQ spectrometer (Thermo Scientific) with electrospray
439 ionization in positive mode (probe temperature, 400°C; cone voltage, 80 V). Single-ion
440 monitoring detected the following compounds: UQ₈ (M⁺ NH₄⁺), m/z 744-745, 6–10 min, scan
441 time of 0.2 s; UQ₁₀ (M⁺ NH₄⁺), m/z 880–881, 10-17 min, scan time of 0.2 s; DMQ₈ (M⁺
442 NH₄⁺), m/z 714–715–10 min, scan time of 0.4 s; DDMQ₈ (M⁺ NH₄⁺), m/z 700-701, 5-8 min,
443 scan time of 0.4 s; OPP (M⁺ NH₄⁺), 656.0-657, 5-9 min, scan time of 0.4 s; compound X (M⁺
444 H⁺), m/z 682-683, 5-10 min, scan time of 0.4 s. MS spectra were recorded between m/z 600
445 and 900 with a scan time of 0.3 s. ECD and MS peak areas were corrected for sample loss
446 during extraction on the basis of the recovery of the UQ₁₀ internal standard and then were
447 normalized to cell wet weight. The peaks of UQ₈ obtained with electrochemical detection or
448 MS detection were quantified with a standard curve of UQ₁₀ as previously described (22).

449

450 **Infections of *G. mellonella* larvae**

451 Larvae of the wax moth *G. mellonella* were purchased from Lombri'carraz SARL,
452 Mery, France. Healthy and uniformly white larvae measuring around 3 cm were selected for
453 infection. The bacteria were grown over night to an OD₆₀₀ of around three. Culture medium
454 was removed by centrifugation and bacteria were diluted in PBS to 10⁸ CFU/mL. Insulin
455 cartridges were sterilized before filling with bacterial solutions. Larvae were injected with 10
456 µL of bacterial suspensions (10⁶ CFU per larva as recommended (24)) using an insulin pen or
457 with 10 µL of PBS only. The precise number of bacteria transferred in injections was
458 determined by spotting serial dilutions onto chocolate agar plates, and counting CFU after
459 growth at 37°C for 48h. Infected larvae were placed in Petri dishes and maintained at 37°C.
460 Survival of larvae was monitored for 6 days by counting the number of dead larvae each day.

461 A cohort of 20 larvae was used per condition, and the experiment was performed twice. As a
462 control, an untreated cohort of larvae was also followed.

463

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469 Aussel for critically reading the paper.

470

471 **Contributions**

472 F.B., F.P., and L.P. conceived the project and its design. K.K., M.H.C. and G.H. conducted
473 experiments and performed data analysis. K.K., C.B. and Y.C. performed experiments on *G.*
474 *mellonella*. L.L. contributed to new reagents (strains). All authors edited the manuscript. L.P.
475 wrote the manuscript. L.P. supervised the project.

476

477 **Abbreviations**

478 The abbreviations used are: MK₈, menaquinone 8; DMK₈ dimethyl-menaquinone 8; OHB, 3-
479 octaprenyl-4-hydroxybenzoic acid; OPP, octaprenylphenol; DMQ₈, C6-demethoxy-
480 ubiquinone 8; DDMQ₈, C1-demethyl-C6-demethoxy-ubiquinone 8; UQ₈, ubiquinone 8;
481 3A4HB, 3-amino-4-hydroxybenzoic acid; pABA, 4-amino-benzoic acid, pA2MBA, 4-amino-
482 2-methoxy-benzoic acid and pA3MBA, 4-amino-3-methoxy-benzoic acid; ECD,
483 electrochemical detection; Tn, transposon; OD₆₀₀, optical density at 600 nm and IPTG,
484 isopropyl-1-thio-β-D-galactopyranoside.

485

486 **Legends**

487 **Figure 1. UQ₈ is the major quinone used by *F. novicida*.** (A) HPLC-ECD analysis of lipid
488 extracts from 1 mg of *E. coli* MG1655 (*E.c.*) and *F. novicida* (*F.n.*) cells grown aerobically in
489 Chamberlain medium with 0.4% (w/v) either glucose or succinate as the sole carbon source.
490 The chromatograms are representative of three independent experiments. The peaks
491 corresponding to UQ₈, DMK₈ and the UQ₁₀ standard are indicated. (B) Mass spectrum of the
492 quinone eluting at 8.30 min from extracts of *F. novicida* grown in the Chamberlain media. H⁺,
493 NH₄⁺ and Na⁺ adducts of UQ₈ are indicated.

494

495 **Figure 2: Proposed UQ₈ biosynthetic pathway in *F. novicida* deduced from the one**
 496 **characterized in *E. coli*.** Corresponding protein IDs in *F. novicida* are indicated in
 497 parenthesis. There is no identified counterpart of UbiD and UbiX in *F. novicida* proteome.
 498 UbiF is only identified in *E. coli* and its functional homolog in *F. novicida* is a Coq7
 499 hydroxylase. Abbreviations used are 4HB, 4-hydroxybenzoic acid; OHB, 3-octaprenyl-4-
 500 hydroxybenzoic acid; OPP, octaprenylphenol; DMQ₈, C6-demethoxy-ubiquinone 8; DDMQ₈,
 501 C1-demethyl-C6-demethoxy-ubiquinone 8; UQ₈, ubiquinone 8.

502

503 **Figure 3. Complementation analysis of *E. coli* UQ₈ biosynthesis mutants with the**
 504 **putative Ubi-proteins from *F. novicida*.** The Δubi *E. coli* mutant strains transformed with
 505 pTrc99a (vec) or pTrc99a encompassing the *ubi_{Fn}* genes were grown over night at 37°C in LB
 506 medium (A) or in M9 minimal medium (B) with 0.4% (w/v) glucose as the sole carbon
 507 source. Expression of the Ubi_{Fn}-proteins was induced by addition of IPTG to a final
 508 concentration of 100 μ M. The *E. coli* wild-type strain MG1655 (WT) transformed with the
 509 pTrc99a empty vector was used as a control. HPLC-ECD analysis of lipid extracts from 1 mg
 510 of cells. The chromatograms are representative of three independent experiments. The peaks
 511 corresponding to OPP, DDMQ₈, DMQ₈, UQ₈, MK₈, DMK₈ and the UQ₁₀ standard are
 512 indicated. (C) Serial dilutions were spotted onto plates containing M9 minimal medium with
 513 0.4% (w/v) either glucose or succinate as the sole carbon source and IPTG (100 μ M final
 514 concentration). The plates were incubated overnight at 37°C.

515

516 **Figure 4. Quantification of cellular UQ₈ contents of Δubi *E. coli* mutant strains**
 517 **expressing the Ubi_{Fn}-proteins.** The Δubi *E. coli* mutant strains transformed with pTrc99a
 518 (vec) or pTrc99a encompassing the *ubi_{Fn}*-genes were grown over night at 37°C in LB medium
 519 (A) or in M9 minimal medium (B) with 0.4% (w/v) glucose as the sole carbon source.
 520 Expression of the Ubi_{Fn}-proteins was described in the legend of the Figure 3. Quantifications
 521 are expressed as the percentages of the control value, which corresponds to the UQ₈ content
 522 of the wild-type strain (n = 3). ****, P < 0.0001 and **, P < 0.005 by unpaired Student's t
 523 test.

524

525 **Figure 5. UQ₈ is essential for the growth of *F. novicida* in respiratory medium.** *F.*
 526 *novicida* (*F.n.*) and transposon mutant of *ubiC_{Fn}* (Tn-*ubiC_{Fn}*) were grown aerobically in

527 Chamberlain medium with 0.4% (w/v) either succinate (**A**) or glucose (**B**) as the sole carbon
 528 source. Growth (average of sixtuplicate growth curves) was followed as the change in the
 529 absorbance at 600 nm in a Tecan plate reader. (**C**) Cellular UQ₈ contents were quantified for
 530 *F.n.* and Tn-*ubiC_{Fn}* according to the materials and methods section. 4HB was added to rescue
 531 the growth and the UQ₈ biosynthesis in Tn-*ubiC_{Fn}*. Quantifications are expressed as pmoles
 532 per mg of cells (n = 3). ****, P < 0.0001 by unpaired Student's t test.

533

534 **Figure 6. Effect of 3A4HB on UQ₈ biosynthesis and growth of *F. novicida*.** (**A**) HPLC-
 535 ECD analysis of lipid extracts from 1 mg of *F. novicida* cells grown aerobically in
 536 Chamberlain medium with 0.4% (w/v) glucose as the sole carbon source and in the presence
 537 of different concentration of 3A4HB solubilized in DMSO (a = DMSO; b = 0.01 mM; c = 0.1
 538 mM; d = 0.25 mM; e = 0.5 mM; f = 1 mM; g = 1 mM 3A4HB + 1 mM 4HB). The
 539 chromatograms are representative of three independent experiments. The peaks corresponding
 540 to UQ₈ and the UQ₁₀ standard, are indicated. Compound X eluting at 6.5 min is marked.
 541 Growth curves for *F. novicida* cultured under aerobic conditions in Chamberlain medium with
 542 0.4% (w/v) either succinate (**B**) or glucose (**C**) as the sole carbon source and in the presence
 543 of either DMSO (control), 1 mM 3A4HB or 1 mM 3A4HB + 100 μM 4HB. The growth for
 544 each condition (average of sixtuplicate growth curves) was followed as the change in the
 545 absorbance at 600 nm in a Tecan plate reader. (**D**) Mass spectrum of compound X eluting
 546 from extracts of *F. novicida* grown in the Chamberlain media with 1 mM of 3A4HB. H⁺ and
 547 Na⁺ adducts corresponding to this molecule were indicated. (**E**) Proposed structure of
 548 compound X in its oxidized form.

549

550 **Figure 7: UQ₈ contributes to the later steps of the infection of *F. novicida* in *G.***
 551 ***mellonella*.** (**A**) The larvae are turning grey/black when infected. (**B**) Survival curve of *G.*
 552 *mellonella* infected with either *F. novicida* (*F.n.*) or the transposon mutant of *ubiC_{Fn}* (Tn-
 553 *ubiC_{Fn}*). ****, P < 0.0001 by Log-rank (Mantel-Cox) test. (**C**) Survival curve of *G. mellonella*
 554 infected with *F. novicida* (*F.n.*) pre-treated or not with 3A4HB (1 mM final concentration).
 555 Each group of *G. mellonella* (n=20) was injected with around 10⁶ CFU/larva and PBS
 556 injection was used as control. n.s. (no significant), P = 0.6670 by Log-rank (Mantel-Cox) test.

557

558 **References**

559

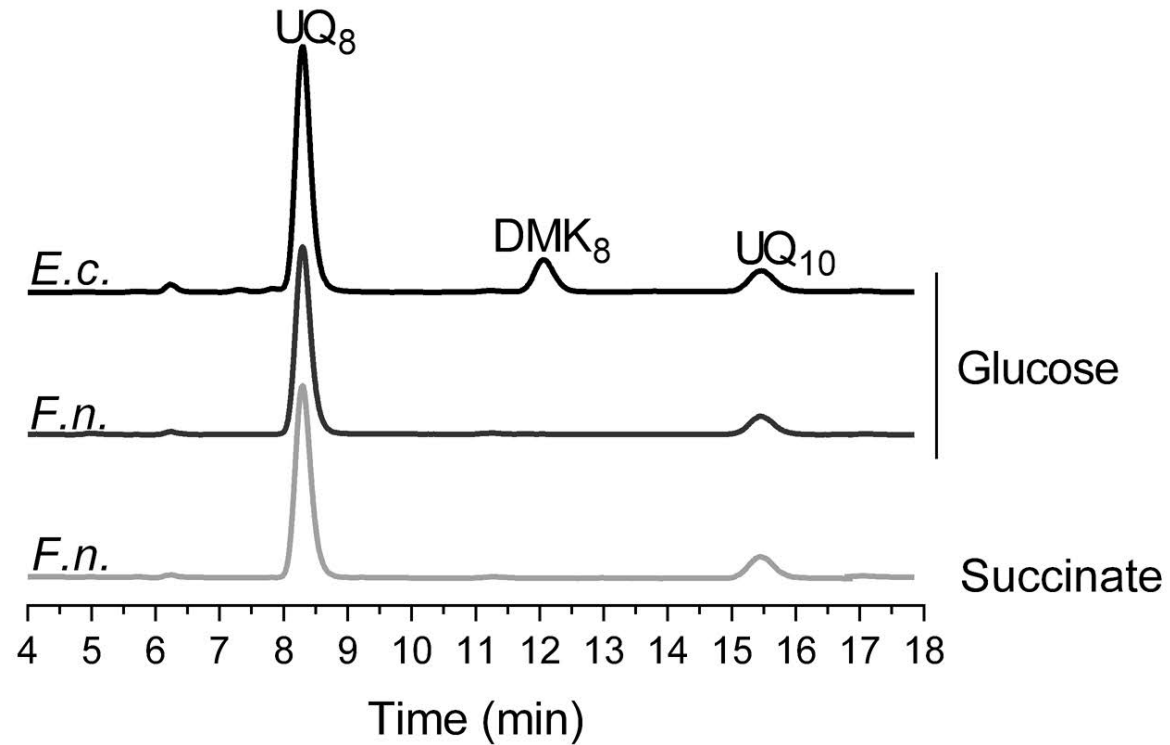
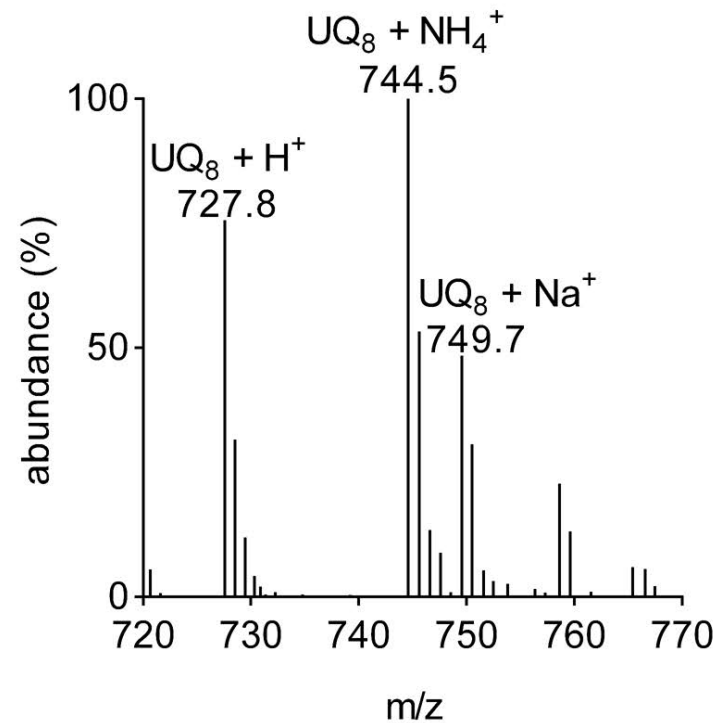
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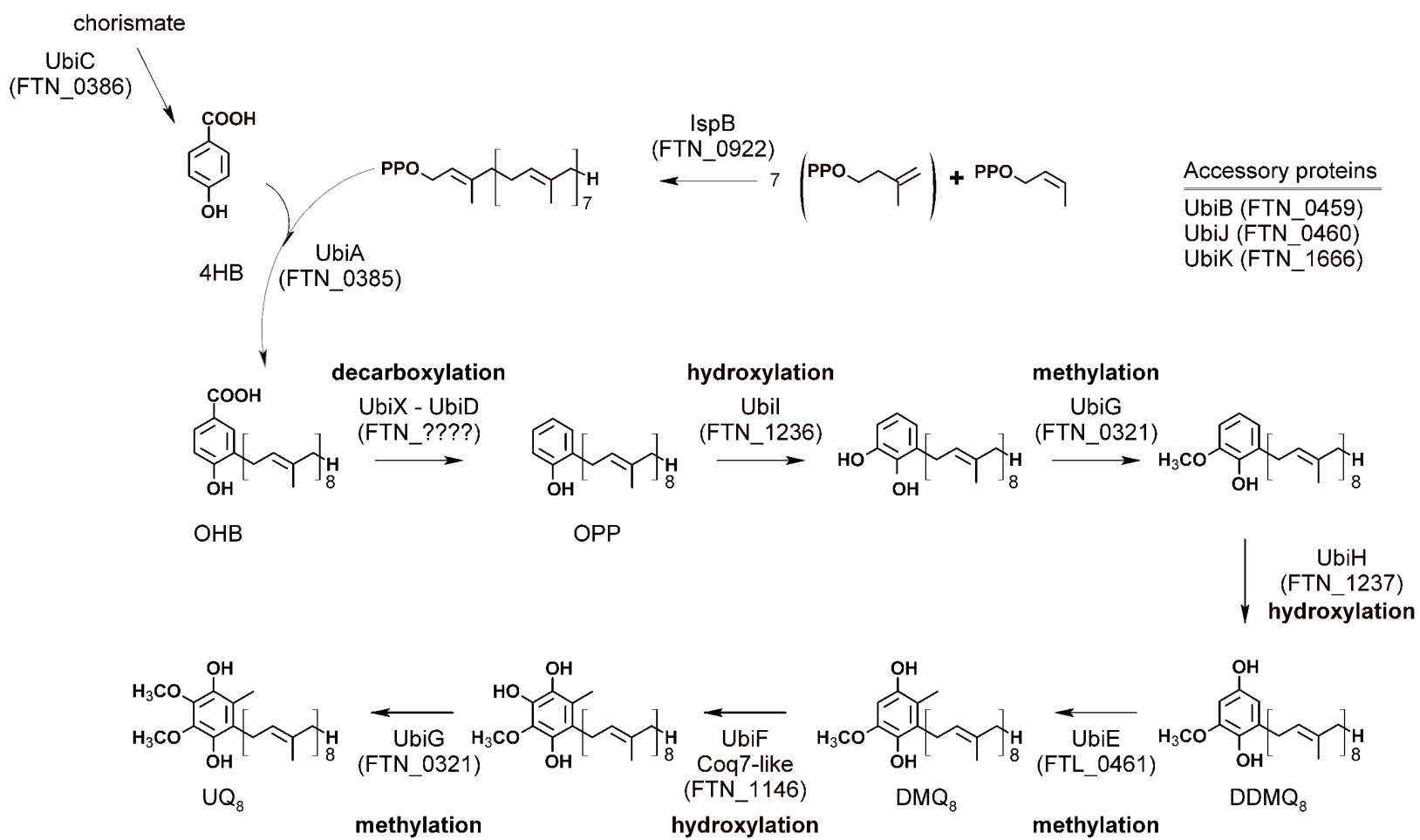
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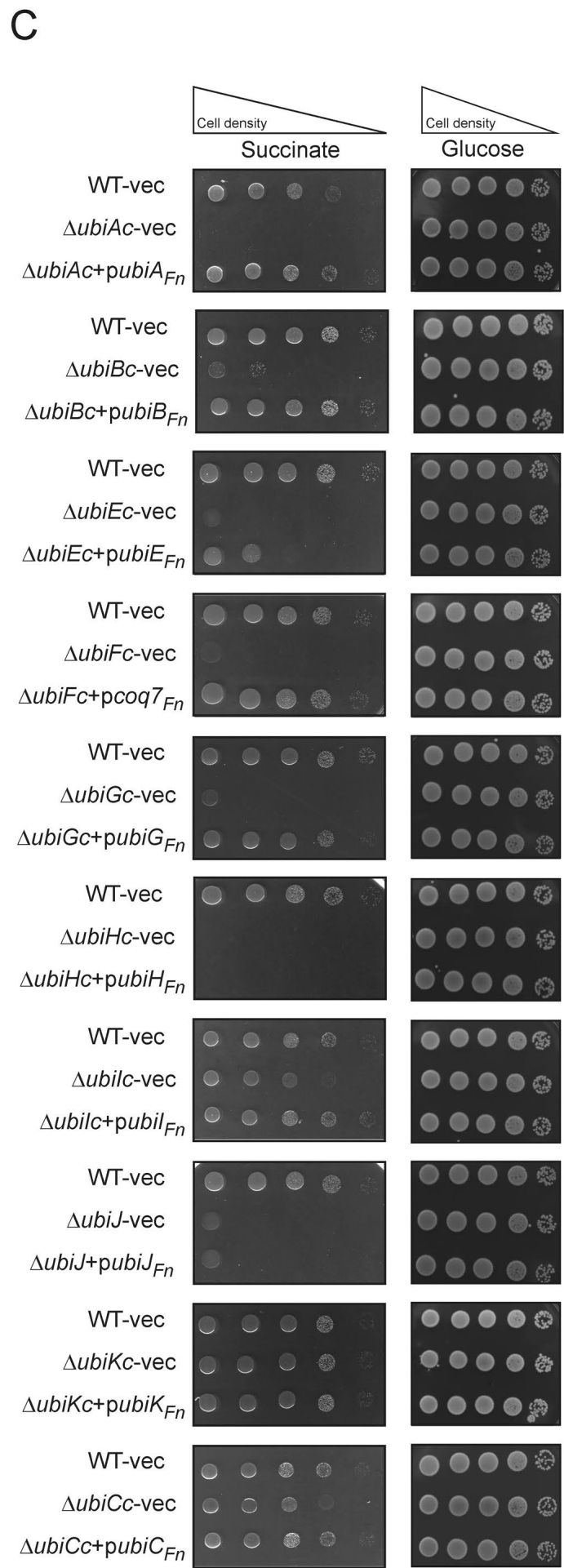
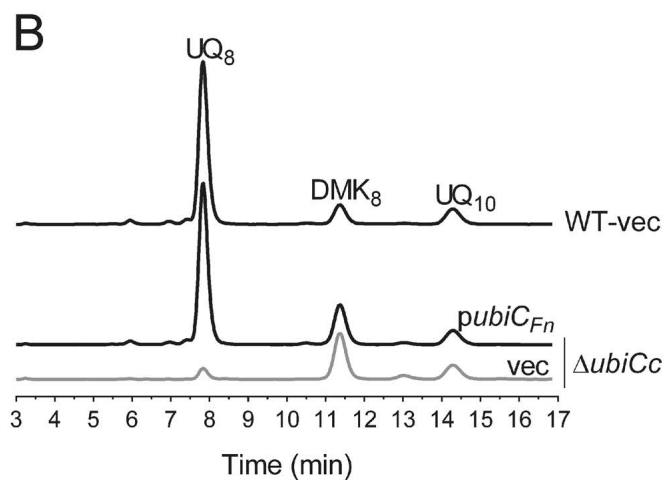
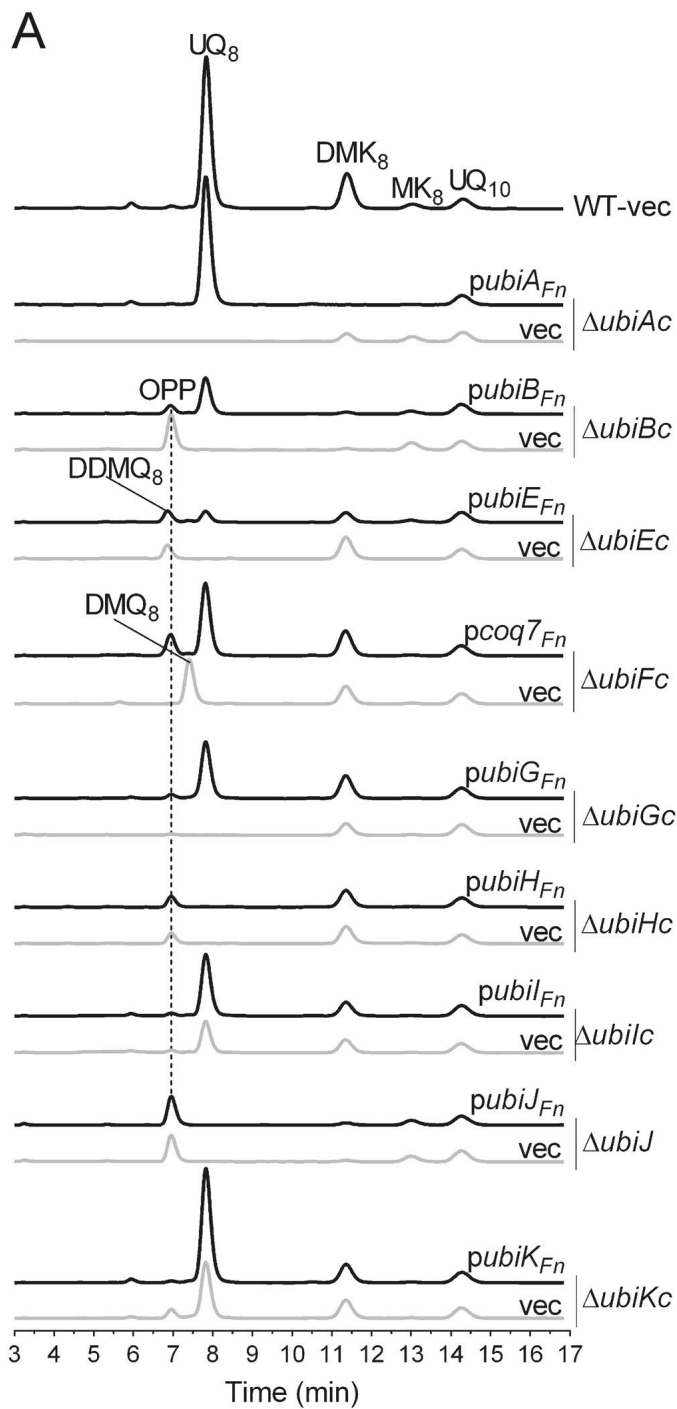
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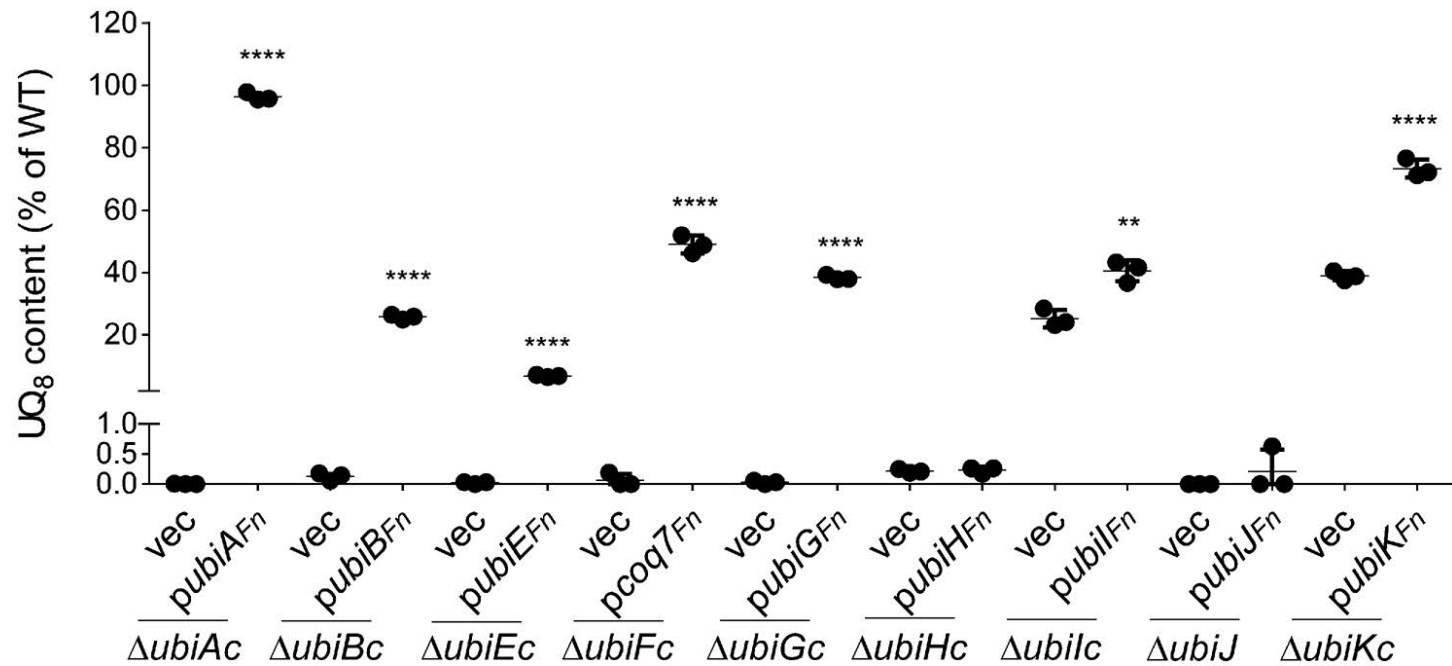
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